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AGGRECANASE-1 AND -2 PEPTIDE SUBSTRATES AND METHODS

FIELD OF THE INVENTION

The present invention describes synthetic peptide substrates of the metalloproteases, aggrecanase-1 and/or -2, suitable for use in assays of enzyme activity. The invention also describes methods using these peptides to discover pharmaceutical agents that modulate these proteases.

BACKGROUND OF THE INVENTION

The disintegrin metalloprotease (or ADAM) family of cell surface proteolytic enzymes is known to play roles in sperm-egg binding and fusion, muscle cell fusion, neurogenesis, modulation of Notch receptor and ligand processing, and processing of the pro-inflammatory cytokine, TNFα (Primakoff and Myles, *Trends Genet* 16:83-87, 2000). The ADAMs have been shown to consist of pre-, pro-, protease, disintegrin-like-, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains. Members of a novel sub-family of the ADAMs, the ADAMTS proteins, lack the transmembrane domain and contain unique thrombospondin motifs, believed to mediate their binding to the extracellular matrix (Tang and Hong, *FEBS Lett.* 445:223-225, 1999). Two members of the ADAMTS family, namely ADAMTS-4 and –5 (also referred to as ADAMTS-11), have been shown to be capable of aggrecan cleavage. Aggrecan is the major proteoglycan of cartilage (Abbaszade *et al.*, *J. Biol. Chem.* 274:23443-23450, 1999; Tortorella *et al.*, *Science* 284:1664-1666, 1999). As a result, these proteins have been implicated in the cartilage damage associated with osteoarthritis and inflammatory joint disease, and have been named "~~A~~aggrecanase-1" (Genbank Accession NM 005099) and "~~A~~aggrecanase-2" (Genbank NM 007038), respectively.

Aggrecanases and MMPs have been shown to cleave aggrecan at a number of different sites (Pratta *et al.*, *J. Biol. Chem.* 275:39096-39102, 2000; Sandy *et al.*, *Biochem. J.* 351:161-166, 2000; Tortorella *et al.*, *J. Biol. Chem.*

275:18566-18573, 2000). Products resulting from cleavage of aggrecan at the site Glu373-Ala374, in the interglobular domain of aggrecan, have been shown to accumulate in synovial fluid of patients with osteoarthritis and inflammatory joint disease (Lohmander *et al.*, *Arthritis Rheum.* 36:1214-22, 1993). Aggrecanase-1 and -2, but not MMPs, are able to cleave aggrecan at this site. A 40 amino acid peptide representing the sequence of aggrecan surrounding the aggrecanase cleavage site (PCT Publication Number WO 00/05256) was able to serve as a substrate for aggrecanase enzymatic activity; however, no peptides less than 40 amino acids in length functioned as suitable substrates for aggrecanase activity, suggesting that shorter substrates, such as substrates of 20 amino acids in length, would not work. Minimum size limits for aggrecanase substrates are consistent with studies suggesting that aggrecanase activity is sensitive to the amino terminal truncation of aggrecan (Horber *et al.*, *Matrix Biol.* 19:533-543, 2000). Glycosylation of the aggrecan substrate has also been shown to affect aggrecanase activity (Pratta *et al.*, *J. Biol. Chem.* 275:39096-39012, 2000).

A sensitive and specific assay for the aggregan degrading metalloproteases, suitable for high-throughput screening, would be helpful in identifying inhibitors of these enzymes for potential therapeutic agents against cartilage damage associated with osteoarthritis and inflammatory joint disease. This invention relates to amino acid peptides shorter than 40 amino acids, unrelated to the aggrecan sequence, but containing aggrecanase sensitive sites, and their use in assays suitable for high throughput screening (HTS) formats.

SUMMARY OF THE INVENTION

The present invention relates to peptides less than 40 amino acids in length having a cleavage site between a glutamic acid on the N-terminal side of the cleavage site and a non-polar or uncharged residue on the C-terminal side of the cleavage site and wherein the peptide is cleavable by an enzyme having an amino acid sequence of SEQ ID NO:8 (truncated a~~A~~ggrecanase-1) and/or SEQ ID NO:9 (truncated a~~A~~ggrecanase-2). In one aspect of this embodiment, the peptide comprises the amino acid sequence of SEQ ID NO:3 and SEQ ID NO:4. Preferably

the peptide is of natural or synthetic origin. In a preferred aspect of this embodiment, the peptide comprises a detectable label selected from the group consisting of ^{125}I, ^{131}I, ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{33}P, a fluorescent dye, or a colorimetric indicator. The peptide preferably also comprises a fluorophore and a quencher or acceptor located at opposite ends of the cleavage site of the peptide. In one embodiment, the peptide further comprises an affinity moiety located at opposite ends of the cleavage site of the peptide.

In another embodiment, the invention relates to a method to identify a compound that inhibits ~~A~~aggrecanase enzymatic activity comprising the steps of: contacting a test compound, an ~~A~~aggrecanase, and a peptide less than 40 amino acids in length wherein the peptide comprises a cleavage site between a glutamic acid on the N-terminal side of the cleavage site and a non-polar or uncharged amino acid residue on the C-terminal side of the cleavage site and wherein the peptide is cleavable by an enzyme having the amino acid sequence of SEQ ID NO:8.; and detecting cleavage of the peptide, wherein inhibition of peptide cleavage in the presence of a test compound indicates compound inhibition of ~~A~~aggrecanase enzymatic activity. In a preferred aspect of this embodiment, the method is performed in a single reaction vessel. Preferably the enzyme is selected from the group consisting of ~~A~~aggrecanase-1 or ~~A~~aggrecanase-2. Preferably the peptide is selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7. Preferably the peptide further comprises a detectable label selected from the group consisting of ^{125}I, ^{131}I, ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{33}P, a fluorescent dye, or a colorimetric indicator. The peptide preferably further comprises a fluorophore and a quencher or acceptor located at opposite ends of the cleavage site of the peptide. In one aspect of this embodiment, the contacting step further comprises a cell expressing the ~~A~~aggrecanase.

metalloprotease activity of aggrecanases-1 or 2

In another aspect of this invention, the invention relates to a method to detect the ability of a compound to inhibit ~~A~~aggrecanase-1 or -2 enzymatic activity comprising the steps of: contacting a test compound,

an ~~A~~aggrecanase secreted by a cell, and a peptide having an amino acid sequence selected from the group consisting of SEQ. ID. NO.:3 or SEQ. ID. NO.:4; incubating the compound, enzyme, and peptide to permit enzymatic cleavage of the peptide; and measuring enzymatic cleavage of the peptide wherein the method is conducted in a single reaction vessel without further manipulation. Preferably the peptide comprises a detectable label selected from the group consisting of ^{125}I, ^{131}I, ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{33}P, a fluorescent dye, or a colorimetric indicator. Also preferably, the peptide comprises a fluorophore and a quencher or acceptor located at opposite ends of the cleavage site of the peptide.

In yet another aspect of this invention, the invention relates to a method to identify a compound capable of inhibiting ~~A~~aggrecanase activity comprising the steps; providing a peptide comprising an affinity moiety, an amino acid sequence selected from a group consisting of SEQ. ID. NO.:3 SEQ. ID. NO.:4 and a detectable label, said affinity moiety and label located on opposite sides of a cleavage site encoded by the amino acid sequence; contacting the peptide with an affinity capture coated solid phase support for sufficient time to bind a portion of the peptide; washing the support to remove unbound peptide; contacting a solution comprising a test compound and functional enzyme with the peptide bound solid phase support for sufficient time to allow enzymatic cleavage of the peptide, thereby releasing the peptide and detectable label into the solution; and measuring changes in the quantity of the detectable label as a result of compound modulation of expected enzymatic function. Preferably the enzyme is selected from the group consisting of ~~A~~aggrecanase-1 and -2. Also preferably the peptide comprises a detectable label selected from the group consisting of ^{125}I, ^{131}I, ^{3}H, ^{14}C, ^{35}S, ^{32}P, ^{33}P, a fluorescent dye, or a colorimetric indicator.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure1 illustrates the domain structures of (A) full-length ~~A~~aggrecanase-1 protein, (B) full-length ~~A~~aggrecanase-2 protein and (C) the

recombinant truncated forms used in a preferred protease assay of this invention.

Figure 2 illustrates the relative activities of ~~A~~aggrecanase-1 (A) and -2 (B) for 56 different fluorescent resonance energy transfer (FRET) peptides, A1 to H7. In Figure 2, every other peptide is numbered.

Figure 3 provides the kinetic analysis of the relative affinities of ~~A~~aggrecanase -2 for cleavage of 2 different peptides

Figure 4 illustrates the use of the ~~A~~aggrecanase-1 and -2 peptide cleavage assays to identify inhibitory compounds. Figure 4A is a comparison of inhibition of ~~A~~aggrecanase-1 proteolytic activity by compounds A and B. Figure 4B provides the IC50 analysis for inhibition of ~~A~~aggrecanase-2 by inhibitory compounds, A, B and C.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of this invention, the invention relates to peptide substrates useful to measure the enzymatic activity of ~~A~~aggrecanase-1 and/or -2 metalloproteases. Using the peptide substrates identified in this invention it is possible to find others that are capable of being cleaved by the preferred truncated ~~A~~aggrecanase-1 and -2 enzymes of this invention. Preferred recombinant truncated forms of human ~~A~~aggrecanase-1 and -2 (i.e., ~~A~~aggrecanase lacking some portion of the complete native sequence), in this invention were creating using the pro- and protease domains and optionally included a FLAG epitope tag, as provided in schematic in Figure 1 (and provided as nucleic acid encoding the truncated ~~A~~aggrecanse, see SEQ ID Nos: 1 and 2 respectively). These recombinant truncated enzymes were produced from Sf9 cells infected with a recombinant baculovirus construct, and purified by affinity chromatography. A number of substrates were identified by screening a collection of 56 potential peptide substrates. Two different peptide sequences were found that were particularly preferred for their ability to be cleaved by truncated ~~A~~aggrecanase-2. One peptide sequence was a good substrate for both truncated ~~A~~aggrecanase-1 and truncated ~~A~~aggrecanase-2. This latter peptide was used to optimize an assay in a format suitable for high throughput screening, which was then used for the

identification of small molecule inhibitors of ~~A~~aggrecanase-1 and -2 as potential therapeutic compounds.

The amino acid sequence of the most preferred peptides is provided in single letter code in Table 1.

Table 1 Relative activities of TRUNCATED AGGRECANASE-1 AND -2 for 2 different FRET peptides

(n.d.= not detectable)

SEQ ID NO:	Peptide name	Peptide sequence	Relative proteolytic Activity	
			Agg-1	Agg-2
3	FasL1	Aedans-E –KELAELRESTS- Dabcyl-K	*	*****
4	29CD23	Aedans-E –ADLSSFKSQEL- Dabcyl-K	n.d.	*****

These peptides and the other peptides of this invention demonstrating ~~A~~aggrecanase substrate activity are useful in assays to discover new pharmaceutical drugs that alter the activity of ~~A~~aggrecanase-1 and/or -2.

The invention also relates to assays using the peptides of this invention to detect compounds that inhibit ~~A~~aggrecanase enzymatic activity. In one aspect of this embodiment, the assay is a homogeneous *in vitro* protein-based assay to detect compound modulation of ~~A~~aggrecanase-1 and/or -2 enzymatic activity.

The term "homogeneous" refers to an assay conducted in a single vessel where there is no further reagent manipulation after the reaction reagents are placed in a vessel. A preferred method comprises the steps of;

1) combining a test compound with an ~~A~~aggrecanase and a peptide substrate,

2) incubating the compound, enzyme, and substrate for a time sufficient to detect substrate cleavage; and

3) detecting substrate cleavage.

In a preferred embodiment, the detecting step comprises detecting a change in the level of substrate cleavage. Preferably the change in the level of substrate cleavage is compared to the change in the level of substrate cleavage in a reaction vessel containing ~~A~~aggrecanase and peptide substrate in the presence of a control test compound that has a known capacity or no capacity to inhibit ~~A~~aggrecanase activity or alternatively in a reaction vessel without test compound.

In a preferred embodiment, the peptide substrate is selected from SEQ ID NO:3 (E5 in Figure 2) or SEQ ID NO:4 (G7 in Figure 2).

Other preferred peptides that can serve as peptides substrates in the assays of this invention for ~~A~~aggrecanase-2 include, but are not limited to:

ID from Fig~~.~~ure 2	Sequence	SEQ ID NO
G1	Aedans-EKARVLAEAADabcyl-Kamide	5
B3	Aedans-EKARVLAEAMDabcyl-Kamide	6
C7	Aedans-ERAEQQRLKSQDLDabcyl-Kamide	7

Still other peptides tested are provided in Table ~~H~~3. In addition, a variety of peptides can also serve as substrates for ~~A~~aggrecanase -1 and/or -2 activity. For example, the present set of peptide substrates was selected by identifying other protease substrates known in the art. The peptides included a collection of substrates for other proteases, as well as a number of sequences corresponding to membrane proximal cleavage sites of various proteins postulated to be released by metalloproteases (including those published by Roghani *et al.*, *J. Biol. Chem.* 274:3531-340, 1999~~)~~ for ADAM9/MDC9). Thus, those of ordinary skill in the art could similarly identify other substrates and test them in the assays of this invention using a truncated ~~A~~aggrecanase as contemplated here.

The term truncated "~~A~~aggrecanase" as used herein refers to a truncated enzyme (as shown in Fig. 1) that displays enzymatic cleavage of a peptide substrate, and for which the corresponding full-length enzyme is known to have the capacity to cleave aggrecan. Efficient cleavage of aggrecan depends on multiple interactions between the enzyme and aggrecan. For example, cleavage depends on an intact N-terminal portion of the substrate, aggrecan (Horber *et al.*, *Matrix Biology* 19:533-543, 2000). Tortorella *et al.* (*J. Biol. Chem.* 275:25791-25797, 2000) showed that cleavage of aggrecan was dependent on the thrombospondin motif in the enzyme, ~~A~~aggrecanase-1, although both full-length and truncated ~~A~~aggrecanase-1 could cleave a peptide substrate (quoted as unpublished data). Currently known ~~A~~aggrecanases are ~~A~~aggrecanase-1 and -2 (Genbank Accession Nos. NM 005099 and NM 007038 respectively). Nucleic acid encoding the truncated versions of these enzymes used in the assays of this invention are provided here as SEQ ID NOS:1 and 2, corresponding to truncated ~~A~~aggrecanase-1 and truncated ~~A~~aggrecanase-2, respectively.

While the ~~A~~aggrecanases used in this invention are truncated forms of a full length native ~~A~~aggrecanase provided by the GenBank citations above, other ~~A~~aggrecanases can be used in this invention as long as they retain their ability to cleave exemplarly peptides SEQ ID NO:3 and SEQ ID NO:4. The ~~A~~aggrecanases used in this invention can be full length, partial, truncated, chimeric or modified enzymes that still retain their ability to cleave the peptides as described in this invention. It has been demonstrated that ~~A~~aggrecanase cleavage sites in aggrecan contain glutamic acid on the N-terminal side of the cleavage site (P1 position) and a non-polar or uncharged residue on the C-terminal side of the cleavage site (P1' position), namely alanine, leucine or glycine (Caterson *et al.*, *Matrix Biology* 19:333-344, 2000; Tortorella *et al.*, *J. Biol. Chem.* 275 18566). As shown later under Kinetic Analysis in Example 2, the truncated ~~A~~aggrecanase-2 used in the assays described here cleaves the peptides of SEQ ID NOS: 3 and 4 between glutamic acid and leucine residues, consistent with the cleavage specificity of aggrecan cleavage sites.

The term "compound" is used herein in connection with a small molecule, preferably an organic molecule that has the potential to disrupt the specific enzymatic activity of the enzyme. For example, but not to limit the scope of the current invention, compounds may include small organics, synthetic or natural amino acid peptides, proteins, synthetic or natural nucleic acid sequences, or any chemical derivatives of the aforementioned. The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington: The Science and Practice of Pharmacy. 1995. Mack Publishing Co. ISBN 0912734051.

The methods described herein are especially useful for high throughput screening (HTS) of compounds to discover compounds that modulate ~~A~~aggrecanase function. The term "high throughput" refers to an assay design that allows easy analysis of multiple samples simultaneously, and capacity for robotic manipulation. Preferred assays are homogeneous assays. Preferred assays also include assay designs that are optimized to reduce reagent usage in order to achieve the analysis desired. The methods described herein demonstrate highly robust performance and good linearity as a function of enzyme concentration and substrate concentration. For example in the assays of the present invention, at appropriately adjusted enzyme and substrate concentrations, the assay was linear for up to four hours. From Figure 4A, it can be seen that for kinetic analysis, the signal-to-noise ratio was effectively infinite, as no change in the background (blank, no enzyme) was observed over the time of the assay. For endpoint measurements, the enzyme and substrate concentrations can be adjusted to achieve the desired signal-to-noise ratio. In the example in Figure 4A, it can be seen that this ratio (control versus blank endpoints) was approximately three. Therefore the amount of reagent

used can be varied to utilize a minimum of expensive reagent, such as a recombinant enzyme.

Examples of assay formats include 96-well or 384-well plates, levitating droplets, and "lab on a chip" microchannel chips used for liquid handling experiments. For example, capillary electrophoresis(CE)-based assays for the activity of proteases have been developed. In this type of system, the assays can be carried out in small volumes (<5µl). Here both the fluorescent-labeled substrate and product can be monitored by laser-induced fluorescence, based on the ability of CE to rapidly separate the two species.

It is well known to those in the art that as miniaturization of plastic molds and liquid handling devices are advanced, or as improved assay devices are designed, that greater numbers of samples may be performed using the design of the present invention. Such new assay designs will not limit the scope of the intended assay.

In another embodiment of the invention, the present invention provides a homogeneous *in vitro* cell-based method to detect compound modulation of ~~A~~aggrecanase enzymatic activity. In this embodiment, the cells express ~~A~~aggrecanase and the peptide substrate and test compound are in contact with ~~A~~aggrecanase. Aggrecanase is preferably released extracellularly. In a preferred embodiment, the ~~A~~aggrecanase is an ~~A~~aggrecanase-1 or an ~~A~~aggrecanase-2. The method comprises the steps of:

1) combining a test compound, a cell expressing ~~A~~aggrecanase, and a peptide substrate; and

2) detecting enzymatic cleavage of the peptide substrate.

Alternatively the assays of this invention could be made non-homogeneous. That is, the assay could be modified to require more than one vessel or a wash step requiring that all events to do not take place in a single reaction sample. Such assays can involve, for example, the immobilization of the substrate peptide. One example is the use of an affinity moiety - affinity capture pair such as streptavidin capture of a biotinylated substrate peptide. Affinity capture pairs are well known in the

art and include, for example, avidin/biotin, antibody capture of a region of the substrate peptide, and polyhistidine/immobilized nickel. A preferred non-homogeneous method comprises the steps of:

1) providing a substrate peptide comprising an affinity moiety, an ~~A~~aggrecanase cleavage site, and a detectable label, said affinity moiety and label located on opposite sides of the cleavage site;

2) contacting the substrate peptide with an affinity capture coated solid phase support for sufficient time to bind a portion of the peptide;

3) washing the support to remove unbound peptide;

4) contacting a solution comprising a test compound and ~~A~~aggrecanase enzyme with the peptide bound solid phase support for sufficient time to allow enzymatic cleavage of the substrate, thereby releasing the substrate and detectable label into the solution; and

5) measuring changes in the quantity of the detectable label as a result of compound modulation of expected ~~A~~aggrecanase enzymatic function.

In one embodiment, the ~~A~~aggrecanase is ~~A~~aggrecanase-1 and/or -2. In another embodiment, the solution is transferred to a reaction vessel prior to the measuring step. The terms solid phase support, affinity capture, unbound versus bound peptide, and the like are all well-known terms to those of ordinary skill in the art to whom this invention pertains and therefore these definitions will not be repeated here.

A change in the quantity of product can be expressed as the total amount of product changing over time (a stop-time assay) or can be kinetic where a change in the enzymatic rate is measured as a function of time. Kinetic assays are preferably measured from the time of initial contact of the enzyme and substrate to a point in time where approximately 50% of the maximum observed product is~~are~~ generated.

The amount of expected ~~A~~aggrecanase enzymatic activity can be determined by running, concurrently or separately, an assay using a compound that does not inhibit enzymatic function (i.e., a blank or a control compound), or with a solvent vehicle that has similar properties as

that used for the test compound but lacks any test compound, such as DMSO, DMF, or isopropyl alcohol.

For cell-based assays, the amount of time necessary for cellular contact with the compound is empirically determined, for example, by running a time course with a known ~~A~~aggrecanase modulator and measuring change as a function of time.

Cells useful in the cell-based ~~A~~aggrecanase assays of this invention are those cells that naturally express ~~A~~aggrecanase, or cells transfected with recombinant ~~A~~aggrecanase. These cells may be immortalized cell lines or primary culture cells from any mammal, preferably murine, rat, rabbit, monkey, chimpanzee, or human.

Methods for detecting compounds that modulate ~~A~~aggrecanase proteolytic activity comprise combining a test compound with an ~~A~~aggrecanase protein and a suitable labeled substrate and detecting the ability of the enzyme to cleave the substrate in the presence of the compound. Enzymatic cleavage can result in release of the label or release of a labeled peptide fragment that can be distinguished from intact labeled peptide. In one example, the substrate is labeled. A variety of methods for exploiting labeled substrates are known in the art. Examples of different types of labeled substrates include, for example, substrate that is radiolabeled (Coolican *et al.*, *J. Biol. Chem.* 261:4170-76, 1986), fluorometric (Twining, *Anal. Biochem.* 143:30-4, 1984) or colorimetric (Buroker-Kilgore and Wang, *Anal. Biochem.* 208:387-392, 1993) substrates.

Radioisotopes useful in the present invention include those well known in the art, specifically ^{125}I, ^{131}I, ^{3}H, ^{14}C, ^{35}S, ^{32}P, and ^{33}P. Radioisotopes are introduced into the peptide by conventional means, such as iodination of a tyrosine residue, phosphorylation of a serine or threonine residue, or incorporation of tritium, carbon or sulfur utilizing radioactive amino acid precursors. Fluorescent resonance energy transfer (FRET)-based methods (Ng and Auld, *Anal. Biochem.* 183:50-6, 1989) can also be used to detect compounds that modulate ~~A~~aggrecanase proteolytic activity. Compounds that are activators will increase the rate of substrate degradation resulting in a reduction in substrate as a function of time. Compounds that are inhibitors

will decrease the rate of substrate degradation and will result in greater remaining substrate as a function of time.

A preferred assay format useful for the method of the present invention is a FRET-based method using peptide substrates that contain a fluorescent donor with either a quencher or acceptor that are separated by a peptide sequence encoding the ~~A~~aggrecanase cleavage site. A fluorescent donor is a fluorogenic compound that can absorb energy and transfers a portion of the energy to another compound. Examples of fluorescent donors suitable for use in the present invention include, but are not limited to, coumarins, xanthene dyes such as fluoresceines, rhodols, and rhodamines, resorufins, cyanine dyes bimanes, acridines, isoindols, dansyl dyes, aminophthalic hydrazides such as luminol and isoluminol derivatives, aminonapthalimides, aminobenzofurans, aminoquinolines, dicanohydroquinones, and europium and terbium complexes and related compounds. A quencher is a compound that reduces the emission from the fluorescent donor when it is appropriately proximally located to the donor. Preferred quenchers do not generally re-emit the energy in the form of fluorescence. Examples of quenching moieties include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, and di- and triphenylmethanes.

A FRET method using a donor/quencher pair measures increased emission from the fluorescent donor as a function of ~~A~~aggrecanase enzymatic activity upon the peptide substrate. Therefore a test compound that antagonizes ~~A~~aggrecanase will generate an emission signal between two control samples – a low (basal) fluorescence from the FRET peptide alone and a higher fluorescence from the FRET peptide digested by the activity of enzymatically active ~~A~~aggrecanase. An acceptor is a fluorescent molecule that absorbs energy from the fluorescent donor and re-emits a portion of the energy as fluorescence. An acceptor is a specific type of quencher that enables a separate mechanism to measure ~~A~~aggrecanase proteolytic efficacy. Methods that use a donor/acceptor pair measure a decrease in acceptor emission as a function of ~~A~~aggrecanase enzymatic activity upon the peptide substrate. Therefore a test compound that antagonizes ~~A~~aggrecanase will generate an emission signal between two control samples – a higher basal

fluorescence from the FRET peptide alone and a lower fluorescence from the FRET peptide digested by the activity of enzymatically active ~~A~~aggrecanase. Examples of acceptors useful in the methods of the present invention include, but are not limited to, coumarins, fluoresceins, rhodols, rhodamines, resorufins, cyanines, difluoroboradiazindacenes, and phthalcyanines. FRET peptides can also be used for zymography (see PCT publication number WO 01/94377 to Fourie et al.) following SDS polyacrylamide gel electrophoresis.

The following examples illustrate the present invention without, however, limiting the same thereto. All references are incorporated herein by reference.

EXAMPLE 1
GENERATION OF TRUNCATED RECOMBINANT ENZYME

Aggrecanase proteins usually comprise: an N-terminal pro-domain and a metalloprotease domain, followed by the disintegrin domain, cysteine-rich domain, epidermal growth factor repeat, thrombospondin repeats and a spacer region, as illustrated in Figure 1. For production of biologically active and soluble ADAMTS proteins (truncated ~~A~~aggrecanase-1 and -2), PCR products containing the pro- and protease domains and a C-terminal FLAG epitope (used for immuno-detection and purification) were cloned into pFastBac1 (GibcoBRL) vectors using standard techniques. The DNA sequences of truncated ~~A~~aggrecanase-1 and -2 used in the methods of this invention are provided as SEQ ID NOS:1 and 2 respectively. The protein sequences corresponding to these DNA sequences are provided as SEQ ID NOS: 8 and 9.

In order to generate large quantities of protein for biological testing and assay development, Sf9 cells were infected with pFastBac (GibcoBRL) containing the coding sequences for truncated ~~A~~aggrecanase-1 or -2.

Recombinant baculovirus for truncated ~~A~~aggrecanase-1 or -2 expression was generated from the pFastBac1 construct described above using the Bac-to-Bac system (Gibco BRL). Sf9 cells were infected with baculovirus and the medium was collected after 72 hours. The medium was concentrated 10-fold by ultrafiltration, and exchanged to TBS (Tris Buffered Saline) by

repeated addition and re-concentration. The supernatant was centrifuged for one hour at 15000 x g, filtered through a 0.45 μM filter to remove debris, and incubated, with mixing, overnight at 4°C with M2-αFlag-agarose (Sigma). The resin was loaded into a column and washed with TBS, followed by elution of the bound material with 0.1M Glycine (pH 3.5) and immediate neutralization by addition of 12.5μl/ml of 2M Tris-HCl, pH 8. The supernatant from the infection (before and after incubation with M2-αFlag-agarose) and fractions from the purification were analyzed by SDS-PAGE followed by staining and Western blotting. By SDS-PAGE, fractions containing the immunopurified truncated ~~A~~aggrecanase-1 or -2 protein contained a protein band with an apparent molecular weight of about 30kDa. Western analysis indicated that the M2αFlag (Sigma) antibody identified a 30kDa band in the infection supernatant before, but not after, anti-FLAG agarose adsorption. The immunoreactive protein was also present in eluted fractions. This protein was then used to test potential substrate peptides.

EXAMPLE 2
FRET ASSAY: PEPTIDE SUBSTRATE SCREENING

Fifty-six different peptides were synthesized to test for protease activity (see Table 3 below). The peptides included a collection of substrates for other proteases, as well as a number of sequences corresponding to membrane proximal cleavage sites of various proteins postulated to be released by metalloproteases (including those published by (Roghani *et al.*, *J. Biol. Chem.* 274:3531-340, 1999) for ADAM9/MDC9). In order to use the principle of fluorescence resonance energy transfer, or FRET, the peptides were labeled at the C-terminus with Dabcyl and at the N-terminus with Aedans (or vice versa). Thus cleavage of the peptides ~~were~~was monitored by the increase in Aedans fluorescence at 460 nm (excitation 360 nm) as a result of the decrease in proximity of the Dabcyl quencher. The assay was performed by diluting the truncated ~~A~~aggrecanase-1 (approximately 2.5 to 5μg of protein, 85 to 167 picomoles, SEQ ID NO:8) or truncated ~~A~~aggrecanase-2 (approximately 0.5 to 1μg of protein, 17 to 33 picomoles, SEQ ID NO:9), in assay buffer (50mM

HEPES pH 7.5, 10mM $CaCl_2$, 0.1M NaCl and 0.05%(w/v) Brij-35 detergent (Sigma).

The reaction was initiated by the addition of peptide substrate to a final concentration of 100uM for truncated ~~A~~aggrecanase-1 and 50uM for truncated ~~A~~agrecanase-2. The assays were typically run for 60 minutes at room temperature and the slope of the kinetic increase in fluorescence was determined to calculate the rate of the reaction.

Figure 2 illustrates the relative activities for the 56 different peptides, A1 to H7 (only every alternate peptide is numbered in Figure 2) expressed in arbitrary, but relative units. Truncated ~~A~~aggrecanase-1 and -2 both showed the highest activity for peptide E5 (FasL1). Truncated ~~A~~aggrecanase-2, but not truncated ~~A~~aggrecanase-1, also showed high activity for cleavage of peptide G7 (29CD23). Peptide D7 (16 amino acids) corresponds to the sequence within aggrecan containing the Glu373-Ala374 ~~A~~aggrecanase cleavage site. Neither truncated ~~A~~aggrecanase-1 nor truncated ~~A~~aggrecanase-2 showed any activity on this peptide, consistent with findings that peptides corresponding to this region of aggrecan, and shorter than 40 amino acids do not function as substrates for aggrecanases (PCT Publication Number WO 00/05256; Horber *et al.*, *Matrix Biology* 19:533-543, 2000).

Peptide E5 (SEQ ID NO:3) was also shown in similar screening assays to be a suitable substrate for the metalloproteases MMP7 and MMP13 (Chemicon, Cat. # CC1059 and CC068 respectively).

Kinetic analysis of the affinity of ~~A~~aggrecanase-1 and -2 for cleavage of 4 different peptides

To confirm the screening assay, ~~A~~aggrecanase-2 was further analyzed for its rate of catalysis using 2 different peptides. The assay was performed by diluting the ~~A~~aggrecanase–2 in assay buffer (50mM HEPES pH 7.5, 10mM $CaCl_2$, 0.1M NaCl and 0.05% Brij-35). As illustrated in Figure 3, the reaction was initiated by the addition of substrate (FasL1 or 29CD23) to different final concentrations for analysis of affinities. The assay was run for 60 minutes at room temperature. Figure 3 illustrates the proteolytic activity (in relative

fluorescence units per minute) as a function of peptide concentration for peptides FasL1 and 29CD23. The curves were fitted to the data with the program Grafit (Erithacus Software Lmited). The results of these analyses are provided in Table 2. The Vmax and Km for each substrate were calculated by non-linear fitting of the data. The cleavage site for ~~A~~aggrecanase-2 within each peptide was determined by LC-MS analysis to be between a glutamic acid and leucine residues in each case, as indicated in Table 2 by a carot within each peptide sequence. These results indicate that the cleavage by the truncated ~~A~~aggrecanase-2 has the same specificity as the full-length enzyme, namely glutamic acid in the P1 position and a non-polar residue in the P1' position. However, these are clearly not the only requirements for efficient cleavage, as a number of the 56 peptides tested have similar residues and were not cleaved by the aggrecanases.

Table 2 K_m and V_m of ~~A~~aggrecanase-2 for peptides

(X = Aedans-E; Z = Dabcyl-K; rfu = relative fluorescence units)

PEPTIDE	*CLEAVAGE SITE*	K_m	V_m
FasL1	X-KELAE^LRESTS-Z	80 µM	2.8 rfu/min
29CD23	X-ADLSSFKSQE^L-Z	40 µM	0.6 rfu/min

Table 3

WELL	SEQUENCE	SEQ. ID NO.
A1	(Aedans)EHSDAVFTDNYTR(Dabcyl)K-amide	10
B1	(Aedans)EAEN(Dabcyl)K-amide	11
C1	(Aedans)EGRHIDNEEDI(Dabcyl)K-amide	12
D1	(Aedans)EGNAFNNLD(Dabcyl)K-amide	13
E1	(Aedans)EYTPNNEIDSF(Dabcyl)K-amide	14
F1	(Aedans)EQLRMKLP(Dabcyl)K-amide	15
G1	(Aedans)EKARVLAEAA(Dabcyl)K-amide	5
H1	(Aedans)ERGFFYTP(Dabcyl)K-amide	16
A2	(Aedans)EVTEGPIP(Dabcyl)K-amide	17
B2	(Aedans)EPLFYEAP(Dabcyl)K-amide	18
C2	(Aedans)ELPMGALP(Dabcyl)K-amide	19
D2	(Aedans)EKPAAFFRL(Dabcyl)K-amide	20
E2	(Aedans)ELYENKPRRPYIL(Dabcyl)K-amide	21
F2	(Aedans)ESEVNLDAEF(Dabcyl)K-amide	22

G2	(Aedans)ESQNYPIVQ(Dabcyl)K-amide	23
H2	(Aedans)EKPIEFFRL(Dabcyl)K-amide	24
A3	(Aedans)EKPAEFFAL(Dabcyl)K-amide	25
B3	(Aedans)EKARVLAEAM(Dabcyl)K-amide	6
C3	(Aedans)EKPAKFFRL(Dabcyl)K-amide	26
D3	R(Aedans)EIPFHLVIHT(Dabcyl)KR	27
E3	(Aedans)EMAPGAVHLPQ(Dabcyl)K-amide	28
F3	(Aedans)EPLAQAVRSSS(Dabcyl)K-amide	29
G3	(Aedans)EPPVAASSLRN(Dabcyl)K-amide	30
H3	(Aedans)EPQIENVKGTE(Dabcyl)K-amide	31
A4	(Aedans)ESLPVQDSSSV(Dabcyl)K-amide	32
B4	(Aedans)EVHHQKLVFFA(Dabcyl)K-amide	33
C4	(Dabcyl)KRGVVNASSRLAK(Aedans)E-amide	34
D4	(Dabcyl)KLVLASSSF(Aedans)E-amide	35
E4	(Dabcyl)KSNRLEASSRSSP(Aedans)E-amide	36

~~Table 3 continued~~

F4	(Aedans)EDEMEE(Abu)ASHLPY(Dabcyl)K-amide	37
G4	(Aedans)EAGPRGMAGQFSH(Dabcyl)K-amide	38
H4	(Dabcyl)KRPLGLAR(Aedans)E-amide	39
A5	(Aedans)EGYYSRDMLV(Dabcyl)K-amide	40
B5	(Aedans)EQKLDKSFSMI(Dabcyl)K-amide	41
C5	(Aedans)EPSAAQTARQHP(Dabcyl)K-amide	42
D5	(Aedans)EPGAQGLPGVG(Dabcyl)K-amide	43
E5	(Aedans)EKELAELRESTS(Dabcyl)K-amide	3
F5	(Dabcyl)GLRTNSFS(Aedans)	44
G5	(Dabcyl)RGVVNASSRLA(Aedans)	45
H5	Ac-ED(Aedans)KPILFFRLGK(Dabcyl)E-amide	46
A6	(Aedans)EMHTASSLEKQIG(Dabcyl)K-amide	47
B6	(Aedans)ERFAQAQQQLP(Dabcyl)K-amide	48
C6	(Aedans)EKKENSFEMQGDQ(Dabcyl)K-amide	49
D6	(Dabcyl)LAQAVRSSSR(Aedans)	50
E6	(Aedans)ERTAAVFRP(Dabcyl)K-amide	51
F6	(Aedans)ERVRRALP(Dabcyl)K-amide	52
G6	(Aedans)ESFPRMFSD(Dabcyl)K-amide	53
H6	(Aedans)EEYLESFLERP(Dabcyl)K-amide	54
A7	(Aedans)ERPKPQQFFGLM(Dabcyl)K-amide	55
B7	(Aedans)EHGDQMAQKSQST(Dabcyl)K-amide	56
C7	(Aedans)ERAEQQRLKSQDL(Dabcyl)K-amide	7
D7	(Aedans)ERNITEGEARGSVIL(Dabcyl)K-amide	57
E7	(Aedans)EAGQRLATAM(Dabcyl)K-amide	58
F7	(Aedans)EVGLMGKRALNS(Dabcyl)K-amide	59
G7	(Aedans)EADLSSFKSQEL(Dabcyl)K-amide	4
H7	(Aedans)EKEDGEARASTS(Dabcyl)K-amide	60

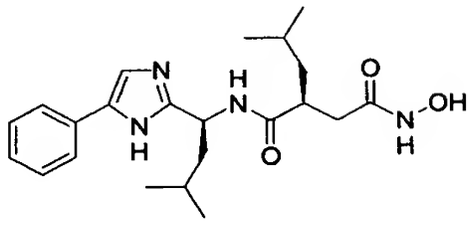
EXAMPLE 3

DRUG SCREENING ASSAY

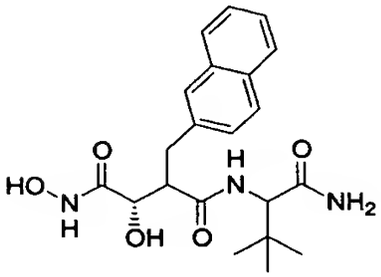
Aggrecanase-1 (2.5 to 5μg of protein, 85 to 167 picomoles) was diluted in assay buffer (50mM HEPES pH 7.5, 10mM $CaCl_2$, 0.1M NaCl, 0.05% Brij-35). Samples were prepared containing putative inhibitors A (Chen et al. Biorg. Med. Chem. Lett. 6(13):1601-1606, 1996) or B (Bailey, et al. Biorg. Med. Chem. Lett. 9(21):3165-3170, 1999), shown below, at a final concentration of 7.5 micromolar. The final %DMSO in the assay was 3% and it was determined experimentally that this concentration was not detrimental to the activity of the enzyme. The reaction was initiated by the addition of FasL1 peptide substrate to a final concentration of 225 μM and readings were taken at one-minute intervals, for a total of 200 minutes at room temperature.

The assay was always performed at enzyme and substrate concentrations where the activity was linearly related to enzyme concentration and where the increase in fluorescence (reaction rate) was linear for at least the time of the assay. From Figure 4A, it can be seen that for kinetic analysis, the signal-to-noise ratio is effectively infinite, as no change in the background (blank, no enzyme) is observed over the time of the assay. For endpoint measurements, the enzyme and substrate concentrations could be adjusted to achieve the desired signal-to-noise ratio. In the example in Figure 4A, it can be seen that this ratio (control versus blank endpoints) was approximately three.

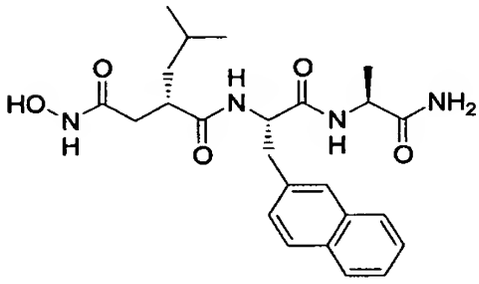
Figure 4A shows that inhibitors A and B completely inhibited ~~A~~aggrecanase-1 enzyme activity (results are comparable to blank [no enzyme]).



Inhibitor A



Inhibitor B



Inhibitor C

IC50 analysis for inhibition of aggrecanase-2 by Inhibitors A, B, and C

Aggrecanase–2 (0.5 to 1μg of protein, 17 to 33 picomoles) was diluted in assay buffer (50mM HEPES pH 7.5, 10mM $CaCl_2$, 0.1M NaCl, 0.05% Brij-35). Samples were prepared containing Inhibitor A, B or C (shown above) at final concentrations ranging from 0.1 to 12.5μM (final DMSO concentration of 1.5%). Duplicate assays were run for each concentration of Inhibitor A, B and C (purchased from Peptides International, TAPI-0, Cat. No. INH 3850-P1) for 60 minutes at room temperature. The reaction was initiated by the addition of FasL1 peptide substrate to a final concentration of 225 μM. The reaction rates over 60 minutes at room temperature, in the absence (control) and presence of various concentrations of the inhibitor, were determined by linear regression of the data points. The reaction rate data in Figure 4B were

fitted by non-linear regression using the program Grafit (Erithacus Software). The IC50s for inhibition of Aggrecanase -2 by Inhibitors A, B and C, were 118 ± 5, 38 ± 8, and 102 ± 23 nM, respectively.

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